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REMARKS

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Pursuant to 37 CFR § 1.121, accompanying this amendment is an appendix listing each of the above amendments to the application and identifying with particularity the changes that have been made.

With respect to the PTO's objection to the drawings under 37 CFR 1.84, twenty-four (24) sheets of corrected formal drawings are submitted herewith to replace the twenty-three (23) sheets of informal drawings as filed. Applicant respectfully requests withdrawal of the objection to the drawings.

The rejection of claim 1 (and claims 2-9 dependent thereon) under 35 U.S.C. § 112, second paragraph, for omitting an essential step is respectfully traversed. Applicant respectfully disagrees that the claimed method of regulating protein kinase activity is lacking an essential step. In particular, regulation of kinase activity does not require measurement of such regulation. Such regulation will occur with or without measurement. For example, applicants have already demonstrated that biliverdin reductase ("BVR") and active fragments thereof regulate protein kinase C activity (see Example 3 at page 67, lines 1-32 and page 71, line 4 to page 73, line 2 with accompanying Figures 11A-C and 12A-C). Given this demonstration, it is not essential to measure the degree of such regulation in performing the recited method. Therefore, the rejection of claims 1-9 for omitting an essential step is improper and should be withdrawn.

The rejection of claim 1 under 35 U.S.C. § 112, first paragraph, for lack of written descriptive support is respectfully traversed in view of the above amendments and the following remarks.

The PTO has taken the position that the present application fails to provide written descriptive support for the claimed method of regulating protein kinase activity on several bases: (1) descriptive support is lacking for use of biliverdin reductase or fragments or variants thereof that do not contain the kinase consensus sequence, (2) descriptive support is lacking for regulating activity of kinases other than protein kinase C. Applicant respectfully disagrees.

With respect to the first basis of rejection, applicant has amended claim 1 to recite that "biliverdin reductase, or an active fragment or variant thereof...." Thus, only fragments or variants that are active in regulating kinase activity can be used in the recited method. The present application describes the functional domains of BVR at page 16, lines 8-33; describes preparation of BVR fragments and identifies exemplary fragments at page 17, line 22 to page 18, line 28; and describes preparation of BVR variants and identifies exemplary variants at page 18, line 29 to page 19, line 24. From the foregoing, it should be appreciated that the present application defines not only specific BVR fragments and variants, including those that have demonstrated efficacy in regulating protein kinase C activity, but also how one of ordinary skill in the art can identify other fragments or variants that can regulate the activity of kinases. For these reasons, one of ordinary skill in the art would appreciate that applicant was in possession of the claimed invention at the time the present application was filed.

With respect to the second basis of the rejection, applicant respectfully submits that the present application provides sufficient written descriptive support for the method of regulating kinase activity. The present application describes the functional domains of BVR at page 16, lines 8-33; and teaches via experimental work with protein kinase C how one of ordinary skill in the art can determine whether BVR (or a fragment or variant thereof) regulates activity of a kinase (see Example 3 at pages 64-75). From the foregoing, it should be appreciated that the present application defines not only that BVR and several active fragments thereof regulate protein kinase C, but also how one of ordinary skill in the art can identify other kinases which are regulated by BVR or its fragments or variants. For these reasons, one of ordinary skill in the art would appreciate that applicant was in possession of the claimed invention at the time the present application was filed.

The rejection of claim 1 (claims 2-5 and 7-9 dependent thereon) under 35 U.S.C. § 112 (first paragraph) for lack of enablement is respectfully traversed. It is the position of the PTO that the specification of the present application is not enabling for regulating kinases other than protein kinase C. Applicant respectfully disagrees.

Accompanying this response is the Declaration of Mahin D. Maines under 37 C.F.R. § 1.132 ("Maines Decl."). In her declaration, Dr. Maines reports her findings that BVR binds to other proteins, as evidenced by the results of a yeast-two hybrid screening procedure which employed BVR as the bait (Maines Decl. ¶¶ 7-10). As a result of such testing and a motif analysis, Dr. Maines identified another kinase that BVR binds,

specifically the Goodpasture Antigen Binding Protein ("GABP") (Maines Decl. ¶ 12 and Exhibit 1 thereto). Based on the facts that (a) BVR binds to GABP and (b) proteins which interact with kinases often do so by binding to the kinase to regulate its activity (see Maines Decl. ¶ 13 and Exhibits 2-4 thereto), it is reasonable to expect that BVR also regulates GABP activity (Maines Decl. ¶ 14). Thus, one of ordinary skill in the art would expect that BVR will regulate GABP activity in phosphorylating the N-terminal region of the Goodpasture antigen (i.e., the non-collagenous domain of the alpha-3 chain of type IV collagen). Whether or not a particular substrate is phosphorylated can be tested with routine skill (see, e.g., Example 2 at pages 55-63 regarding autophosphorylation of BVR).

The PTO has also cited to Salim et al., J. Biol. Chem. 276(14):10929-10934 (2001) ("Salim") for the proposition that BVR is a serine/threonine phosphoprotein, but not a tyrosine phosphoprotein. The PTO's reliance on Salim in this regard is misplaced, because Salim does not demonstrate that BVR is not a tyrosine phosphoprotein. Salim is silent in this regard. Moreover, contrary to the PTO's position is the data reported in the present application, which demonstrates that BVR is a serine/threonine/tyrosine phosphoprotein (see Example 2 at page 59, line 1 to page 60, line 20 and Figures 7-8). Therefore, one of ordinary skill in the art would expect that BVR (or its active fragments or variants) will regulate the activity of serine/threonine kinases as well as tyrosine kinases.

For these reasons, the rejection of claim 1 as well as claims 2-5 and 7-9 dependent thereon for lack of enablement is improper and should be withdrawn.

The rejection of claim 8 under 35 U.S.C. § 112, first paragraph, lack of written descriptive support is respectfully traversed. The PTO has asserted that the present application fails to provide sufficient written descriptive support for the claimed invention because the specification fails to describe in vivo use of BVR (or active fragments or variants thereof) for purposes of regulating kinase activities. Applicant respectfully disagrees.

The present application identifies a number of techniques for introducing BVR into a patient, including (among others) liposome delivery, BVR-conjugates, and BVR chimera (see page 42, line 7 to page 44, line 4). These approaches can be utilized to deliver BVR (or active fragments or variants thereof) via various administration routes (see page 45, lines 24-32). By way of example, such BVR administration can be performed for purposes of regulating PKC activity in any one of a number of conditions in which PKC has a demonstrated role in disease pathology (see page 24, line 30 to page 25, line 2). Thus, the present application provides a written description of in vivo uses for BVR or fragments or

variants thereof. Moreover, because of applicant's demonstration that BVR is both a serine/threonine and a tyrosine phosphoprotein, one or ordinary skill in the art would expect BVR to regulate the activity of other kinases other than PKC. This is likely to be true given applicant's subsequent demonstration of BVR binding to GABP (see supra discussion of Maines Decl.). For these reasons, the present application provides sufficient written descriptive support for the presently claimed invention and one of ordinary skill in the art would understand that the applicant was in possession of the presently claimed invention.

The rejection of claim 8 under 35 U.S.C. § 112, first paragraph, for lack of enablement is respectfully traversed. The PTO has taken the position that the present application is not enabling for in vivo regulation of protein kinase activity. Applicant respectfully disagrees.

It is well settled that there is no requirement under 35 U.S.C. that an application contain in vivo data. In this regard, Cross v. Iizuka, 753 F.2d 1040, 224 USPQ 739 (Fed. Cir. 1985) is constructive:

> Opinions of our predecessor court have recognized the fact that pharmacological testing of animals is a screening procedure for testing new drugs for practical utility. See, e.g., In re Jolles, 628 F.2d 1322, 1327, 206 USPQ (BNA) 885, 890 (CCPA 1980). This in vivo testing is but an intermediate link in a screening chain which may eventually lead to the use of the drug as a therapeutic agent in humans. We perceive no insurmountable difficulty, under appropriate circumstances, in finding that the first link in the screening chain, in vitro testing, may establish a practical utility for the compound in question. Successful in vitro testing will marshal resources and direct the expenditure of effort to further in vivo testing of the most potent compounds, thereby providing an immediate benefit to the public, analogous to the benefit provided by the showing of an in vivo utility. Cf. Nelson, 626 F.2d at 856, 206 USPQ (BNA) at 883.

Id. at 1051, 224 USPQ at 747-48. Likewise, there is no reason why in vitro data would not be useful in satisfying the enablement requirement of 35 U.S.C. § 112 for in vivo methods of use as presently claimed.

In the presently claimed invention, regulation of protein kinase activity is recited. Given that protein kinase activity can be regulated in vitro (see Eample 3) and applicant has demonstrated that BVR binds to other kinases (see supra discussion of Maines Decl.), there is no reason to question whether protein kinases can be regulated in vivo.

As noted above, BVR has a demonstrated role in regulating PKC activity in vitro. BVR is not the first compound ever disclosed to have a regulatory effect on PKC. In fact, much is known in the art about the cellular activities of PKC and its regulatory elements. For example, it is widely known that PKC plays an important role in cell-cell signaling, gene expression, the control of cell differentiation and growth, cancer development, and functioning of the central nervous system. Targeting the activity of PKC for regulatory effects is also widely known in the art. In particular, PKC inhibitors have been shown to prevent the damage seen in focal and central ischemic brain injury and brain edema (Hara et al., J. Cereb. Blood Flow Metab. 10:646-653 (1990) (copy attached as Exhibit A). It is also known that inhibitors of PKC are effective in preventing tumor growth in animals (Meyer et al., Int. J. Cancer 43:851-856 (1989) (copy attached as Exhibit B).

Based on the in vitro results demonstrated in the present application and supported by the Maines Decl., one of ordinary skill in the would expect BVR (or active fragments or variants thereof) to be useful in regulating kinase activity in vivo because in vivo delivery of kinase inhibitors has previously been demonstrated and applicant's in vitro data is predictive of in vivo success. Therefore, the rejection of claim 8 for lack of enablement is improper and should be withdrawn.

In view of all of the foregoing, applicant submits that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: August 20, 2002

Edwin V. Merkel
Registration No. 40,087

Nixon Peabody LLP
Clinton Square, P.O. Box 31051
Rochester, New York 14603-1051
Telephone: (585) 263-1128
Facsimile: (585) 263-1600

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Date 8/20/02 | Wendy L. Barry

In reference to the amendments made herein to the claims, additions appear as double-underlined text while deletions appear as strikeout text, as indicated below:

In the Specification:

At page 2, line 16 to page 3, line 2:

BVR was previously thought to be simply a house-keeping enzyme found in most mammalian cells in excess of, or in disproportionate levels to, heme oxygenase isozymes (Ewing et al., J. Neurochem. 61:1015-1023 (1993)). Yet it has the above-noted ~~noted~~ unique and uncommon properties. Examination of the primary structure of human BVR, which recently became available (Maines et al., Eur. J. Biochem. 235:372-381 (1996)), revealed the presence of consensus sequences that are conserved in protein kinases, the most notable one being the Gly.Xaa.Gly[17].Xaa.Xaa.Gly motif near the N terminus of the protein that is found invariably in all kinases (Kamps et al., Nature 310:589-592 (1984); Hunter et al., Ann. Rev. Biochem. 54:897-930 (1985); Schlessinger, Trend. Biochem. Sci. 13:443-447 (1988); Hanks et al., Science 241:42-52 (1988); Yarden et al., Annu. Rev. Biochem. 57:443-478 (1988); Hanks et al., Methods Enzymol. 200:38-62 (1991)). A valine residue is present in BVR just 2 positions downstream from the last glycine. A valine residue is invariant at the corresponding position, as in BVR, in the family of kinases that phosphorylate G-protein coupled receptors (Garcia-Bustos et al., Biochim. Biophys. ACTA 1071:83-101 (1991)). Database search results also identified additional similarities with PKGs, including a cluster of charged residues (Lys[224].Arg.Asn.Arg) in the carboxy terminus of BVR. Such clusters are a characteristic of the nuclear localization signal ("NLS") (Garcia-Bustos et al., Biochim. Biophys. ACTA 1071:83-101 (1991)).

At page 4, lines 26-30:

Figure ~~Figures~~ 1 is an image of an SDS-PAGE of hBVR subjected to immunoblotting. Phosphorylation molecular weight markers are shown on the left side (panel a) and hBVR immunoblotting on the right side (panel b). Immunoblotting used 2 μg of hBVR with a mixture (2 μg/ml each) of anti-phosphotyrosine, anti-phosphothreonine, and anti-phosphoserine ("anti-phospho mix").

APPENDIX
Page 2 of 2

At page 6, lines 1-5:

Figures 7A-D are ~~Figure 7 is an image~~ images of ~~an~~ SDS-PAGE ~~immunoblot~~ immunoblots which ~~illustrates~~ illustrate that biliverdin reductase is a serine-, threonine-, tyrosine-phosphoprotein. Purified rat liver BVR (1-3 μg/ml each) was subjected to SDS-PAGE and immunoblotting with the (anti-phospho mix (2 μg/ml each) (Figure 7A ~~panel a~~), anti-phosphotyrosine (Figure 7B ~~panel b~~), anti-phosphoserine (Figure 7C ~~panel c~~), and anti-phosphothreonine (Figure 7D ~~panel c~~).

At page 6, line 30 to page 7, line 5:

Figures 12A-C are graphs illustrating the effect of various rBVR fragments on PKC activity. Protein kinase C was incubated at 30°C with buffer or with the indicated peptides (50 μM) for 15 min prior to addition to a kinase assay using MBP as substrate. Figure 12A depicts the relative activity for each sample normalizing to that of the PKC and buffer control value. Figures 12B-C are the kinetic analyses of PKC with respect to substrate MBP (Figure 12B) and ATP (Figure 12C) in the presence or absence of BVR peptide fragments (50 μM). PKCI corresponds to SEQ. ID. No. 29, BVR1 peptide corresponds to SEQ. ID. No. 34, and BVR2 peptide corresponds to SEQ. ID. No. 19.

In the Claims:

1. (Amended) A method of regulating protein kinase activity comprising:

contacting a protein kinase with biliverdin reductase, or an active fragment ~~fragments~~ or ~~variants~~ variant thereof, under conditions effective to regulate protein kinase activity.

4. (Amended) The method according to claim ~~2~~ 1, wherein the biliverdin reductase is rat or human biliverdin reductase.

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Staurosporine, a Novel Protein Kinase C Inhibitor, Prevents Postischemic Neuronal Damage in the Gerbil and Rat

Hideaki Hara, Hiroshi Onodera, Mikio Yoshidomi, *Yuzuru Matsuda, and Kyuya Kogure

*Department of Neurology, Institute of Brain Diseases, Tohoku University School of Medicine, Sendai, and *Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan*

Summary: The protective effects of protein kinase inhibitors and a calmodulin kinase inhibitor (W-7) against ischemic neuronal damage were examined in the CA_1 subfield of the hippocampus. Staurosporine, KT5720, and KT5822 were used as inhibitors of protein kinase C (PKC), cyclic AMP–dependent protein kinase, and cyclic GMP–dependent protein kinase, respectively. All test compounds were injected topically into the CA_1 subfield of the hippocampus. In the gerbil ischemia model, staurosporine (0.1–10 ng) administered 30 min before ischemia prevented neuronal damage in a dose-dependent manner. However, KT5720, KT5822, and W-7 were ineffective, even at a dose of 10 ng. In the rat ischemia model, staurosporine (10 ng) also prevented neuronal damage when administered before ischemic insult, although staurosporine administered 10 or 180 min after recirculation was ineffective. These results suggest the involvement of PKC in CA_1 pyramidal cell death after ischemia and that the fate of vulnerable CA_1 pyramidal cells through PKC-mediated processes could be determined during the early recirculation period. **Key Words:** Cerebral ischemia—Cyclic nucleotide–dependent protein kinase—Hippocampus—Protein kinase C—Selective vulnerability—Staurosporine.

CA_1 pyramidal cells in the hippocampus are selectively vulnerable to transient ischemic insult. Neuronal death of these cells occurs after an interval of 1 or 2 days following recirculation, during which time no energy crisis or morphological changes are observed ("delayed" neuronal death; Kirino, 1982; Pulsinelli et al., 1982). Some studies have indicated that an excessive influx of calcium and/or an abnormal release of transmitters, such as glutamate, during and after ischemia is responsible for the neuronal damage of CA_1 neurons (Benveniste et al., 1984; Onodera et al., 1986). This hypothesis is based on the findings obtained using selective glutamate receptor antagonists (Simon et al., 1984; Gill et al., 1987) and inhibitors of glutamate-operated calcium channels (Izumiyama and Kogure, 1988).

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent enzyme highly concentrated in the brain (Kikkawa et al., 1982). The activation of PKC plays a critical role in neurotransmitter release and synaptic plasticity (Malenka et al., 1986; Nishizuka, 1986). Immunohistochemical localization of PKC (Saito et al., 1988) and phorbol ester autoradiography (Worley et al., 1986) revealed that the hippocampus contains a high concentration of PKC molecules in the brain, with the highest density in the CA_1 subfield, where selective pyramidal cell death is observed after ischemia. Hydrolysis of phosphoinositides into diacylglycerol and inositol phosphates, which was observed after ischemia (Abe et al., 1987), may modulate protein phosphorylation via PKC (Gonzales et al., 1987). We reported that phorbol ester binding in the dendritic fields of the CA_1 subregion increased during early recirculation and reached a maximum 6–12 h after ischemia (Onodera et al., 1989). Consequently, this enzyme may play a pivotal role in the postischemic modulation

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Address correspondence and reprint requests to Dr. H. Hara at Kanebo Ltd., Pharmaceuticals Research Center, 5-90 Tomobuchi-cho I-chome, Miyakojima-ku, Osaka 534, Japan.

Abbreviations used: MAP2, microtubule-associated protein 2; PKC, protein kinase C.

of synaptic efficacy in the hippocampal formation and neuronal death of CA_1 pyramidal cells. However, the postischemic changes and the roles of intracellular signal transduction systems, such as PKC, cyclic neucleotide–dependent protein kinases, and calmodulin, are not well known.

The aim of the present study was to clarify the contribution of protein kinases to the ischemic death of CA_1 pyramidal cells. The protective effects of protein kinase inhibitors and calmodulin kinase inhibitor W-7 (Hidaka et al., 1978) against ischemic neuronal damage were estimated in the CA_1 subfield of the hippocampus. We estimated the neuronal damage by means of histopathology and immunohistochemistry of microtuble-associated protein 2 (MAP2), a marker protein of dendrites (Matus et al., 1981; Caceres et al., 1983; Bernhardt and Matus, 1984). Staurosporine (Tamaoki et al., 1986), KT5720 (Kase et al., 1987), and KT5822 (Kase et al., 1987) were used as inhibitors of PKC, cyclic AMP–dependent protein kinase, and cyclic GMP–dependent protein kinase, respectively. Mongolian gerbils have been commonly used to estimate the protective effects of drugs against ischemic CA_1 pyramidal cell damage (Kirino, 1982; Kirino et al., 1986*a*,*b*; Gill et al., 1987; Izumiyama and Kogure, 1988), although involvement of the hippocampus in the seizure activities characteristic of this animal has been reported (Paul et al., 1981; Peterson et al., 1985). Interestingly, the protective effect of drugs against ischemic CA_1 damage is more pronounced in gerbil models than rat models. Drugs with antiepileptic potency may be beneficial to protect against CA_1 pyramidal cell damage in the gerbil ischemia model (Kirino et al., 1986*b*; Taft et al., 1989). Therefore, we used both rat and gerbil ischemia models to estimate the protective role of staurosporine against ischemic death of CA_1 pyramidal cells.

MATERIALS AND METHODS

Gerbil model

Male Mongolian gerbils weighing 60–80 g each were divided into eight groups at random; no selection criteria were used for attributing the animals to the various groups. The animals were anesthetized (2% halothane, 70% N_2O, 30% O_2) and mounted on a stereotaxic apparatus. Thereafter anesthesia was maintained with 1% halothane. One microliter of vehicle (5% dimethyl sulfoxide in saline) or test compounds was stereotaxically administered into the bilateral CA_1 subfield of the hippocampus over a period of 10 min, according to the atlas of Loskota et al. (1974). The site was 1.8 mm posterior to bregma, 2.0 mm lateral to the midline, and 1.4 mm into the dural surface. In sham-operated animals, the cannulation was made but neither injection nor ischemia was induced. Thirty minutes after the righting reflex was regained, both common carotid arteries were exposed under 1% halothane anesthesia. After completion of the surgical procedures, halothane was discontinued, and 2 min later both carotid arteries were clipped for 5 min. The body temperature of the animals was maintained at 37°C using a heating pad with thermostat and a heating lamp during the operation period and until righting reflex reappeared, because hypothermia acts protectively against the postischemic neuronal damage (Clifton et al., 1989; Möller et al., 1989). Seven days after ischemia, the animals were perfused transcardially with heparinized saline followed by 10% formalin under deep pentobarbital anesthesia. The brains were removed and embedded in paraffin using standard procedures. Coronal sections (5 μm thick) containing the needle tract were stained with cresyl violet, and the number of neurons in the linear length (1 mm) of the stratum pyramidale of the hippocampal CA_1 subfield (neuronal cell density) was counted in each specimen, according to the method of Kirino et al., (1986*a*). The linear length of the CA_1 subfield was measured with a digitizer (Wacom Co.).

Immunohistochemistry

Some coronal sections, 5 μm thick, in the gerbil model were immunostained with a monoclonal antibody to MAP2 (1:1,000; Sigma, St. Louis, MO, U.S.A.) for 2 h at room temperature. An anti-mouse avidin-biotinylated horseradish peroxidase kit was used according to the supplier's recommendations (Vector Labs) and the sections reacted with 3,3-diaminobenzidine tetrahydrochloride. Staurosporine (10 ng/body) was administered 30 min prior to ischemia.

Rat model

Male Wistar rats (250–300 g) were used and divided into six groups at random. Transient forebrain ischemia was induced according to the method of Pulsinelli et al. (1982). Briefly, the animals were anesthetized with pentobarbital (50 mg/kg i.p.) and both vertebral arteries were electrocauterized. The next day the common carotid arteries were exposed under 1% halothane in a mixture of 70% N_2O and 30% O_2. After completion of the surgical procedure, the halothane was discontinued, and 2 min later both common carotid arteries were clamped using aneurysm clips for 20 min. Topical injection into the CA_1 subfield (2 μl) of vehicle (5% dimethyl sulfoxide in saline) or staurosporine dissolved in the vehicle was carried out unilaterally over a period of 4 min, and the needle was left for 2 min. Staurosporine was administered before ischemia (30 min) or after ischemia (10 or 180 min) through a 27-gauge stainless-steel needle. Coordination was as follows: 3.6 mm posterior to bregma, 1.9 mm lateral to the midline, and 2.8 mm from the dural surface, according to the atlas of Paxinos and Watson (1986). The body temperature of the animals was maintained at 37°C using a heating pad with thermostat and a heating lamp during the operation period and until righting reflex reappeared. The animals were perfusion-fixed and stained 7 days after ischemia as described above.

Cellular distribution

PC12 cells were collected 15 min and 2, 8, and 24 h after 1-h incubation with 200 n*M* staurosporine and washed by centrifugation. The resultant pellet was dissolved in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

buffer (pH 7.4) and sonicated for 20 s with a Tomy Seiko Ultrasonic Disruptor (UR-200P). The preparation was centrifuged for 10 min at 19,000 *g*. The staurosporine present in the supernatant was extracted with chloroform/methanol (9:1, vol/vol). The extracts were concentrated in vacuo and dissolved in 100 μl of dimethyl sulfoxide followed by dilution with *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid buffer. The amount of staurosporine was determined with a fluorescence spectrophotometer (F-3000; Hitachi) at an excitation wavelength of 290 nm and emission wavelength of 300–550 nm. The staurosporine in the culture medium was also extracted and measured. Positive control reaction was done in a similar manner with fluorescein diacetate, which is known to cross the cell membrane.

Test compounds

Staurosporine, KT5720, and KT5822 were gifts from Kyowa Hakko Kogyo Co. Ltd. (Japan). W-7 and monoclonal antibody of MAP2 were obtained from Sigma.

Statistics

Statistical comparisons were made by the Kruskal-Wallis one-way analysis of variance, two-tailed Mann-Whitney U test, and paired *t* test.

RESULTS

Gerbil model (Table 1, Fig. 1)

In the sham-operated group (cannulated in the CA_1 without ischemia or injection), the neuronal cell density of the CA_1 subfield was 246 ± 23/mm (mean ± SD, n = 12). In the ischemia group (with vehicle injection), the number of CA_1 pyramidal cells was markedly decreased (81 ± 47/mm, n = 13), which was statistically significant ($p < 0.01$). Staurosporine (0.1, 1, and 10 ng) administered 30 min preceding transient ischemia, in a dose-dependent manner significantly ($p < 0.01$) protected against pyramidal cell loss in the CA_1 subfield at a concentration of 1 and 10 ng. On the other hand, KT5720, KT5822, and W-7 had no protective effect on postischemic CA_1 pyramidal cell death.

TABLE 1. *Effects of various protein kinase inhibitors on delayed neuronal death of hippocampal CA_1 neurons in gerbils*

Treatment	Dose (ng)	n	Neuronal density (n/mm)
Sham operation	—	12	246 ± 23[a]
Vehicle	—	13	81 ± 47
Staurosporine	0.1	7	117 ± 55
	1	10	166 ± 78[a]
	10	10	201 ± 95[a]
KT5720	10	9	91 ± 69
KT5822	10	10	109 ± 78
W-7	10	9	111 ± 82

Various protein kinase inhibitors were injected topically into the CA_1 subfield of the hippocampus 30 min prior to 5-min forebrain ischemia. Values are means ± SD.

[a] $p < 0.01$ vs. vehicle (Kruskal-Wallis test and two-tailed Mann-Whitney U test).

Immunohistochemistry (Fig. 2)

MAP2 immunohistochemistry, which provides a marker protein of dendrites, revealed that MAP2 immunoreactivity in hippocampus pretreated with staurosporine was similar to that of sham-operated animals, although animals without staurosporine had a marked decrease in MAP2 immunoreactivity.

Rat model (Table 2, Fig. 3)

In the sham-operated group, the neuronal cell densities of the CA_1 subfield were 165 ± 18/mm (ipsilateral side, n = 6) and 174 ± 13/mm (contralateral side, n = 6). In the vehicle-treated group subjected to 20 min of ischemia, the neuronal cell density of the CA_1 subfield ipsilateral to the vehicle injection decreased to 28 ± 37/mm (n = 9), and the density of the contralateral CA_1 subfield was 22 ± 21/mm (n = 9). There was no significant difference in the neuronal cell density between ipsilateral and contralateral to the vehicle treatment. The unilateral injection of staurosporine at a dose of 10 ng 30 min before induction of ischemia reduced the damage of the CA_1 pyramidal cells ipsilateral to the injection (Fig. 3). The CA_1 pyramidal cell density ipsilateral to the staurosporine injection was 78 ± 60/mm (n = 9), which was statistically significant ($p < 0.001$) compared to the vehicle-treated ischemia group (ipsilateral to the vehicle injection). However, the CA_1 pyramidal cell density contralateral to the staurosporine treatment was not different from that in the vehicle-treated ischemia group both ipsi- and contralateral to the injection. In contrast to the protective effect of staurosporine administered 30 min before induction of ischemia, staurosporine treatments 10 or 180 min after recirculation failed to prevent the loss of CA_1 pyramidal cells (Table 2).

Cellular distribution (Fig. 4).

The intracellular amount of staurosporine reached a peak 15 min after the 1-h incubation, with little change thereafter. The intracellular concentration was >900 n*M* (packed cell volume). Similar changes were observed in the extracellular amount of staurosporine, except that the peak was reached 2 h after incubation.

DISCUSSION

Staurosporine administered topically to the CA_1 subfield preceding a brief period of forebrain ischemia was effective in protecting against CA_1 pyramidal cell damage in both rats and gerbils. It penetrated into the cells. Staurosporine, a microbial alkaloid that has been known to have antifungal activity (Omura et al., 1977), was found to inhibit

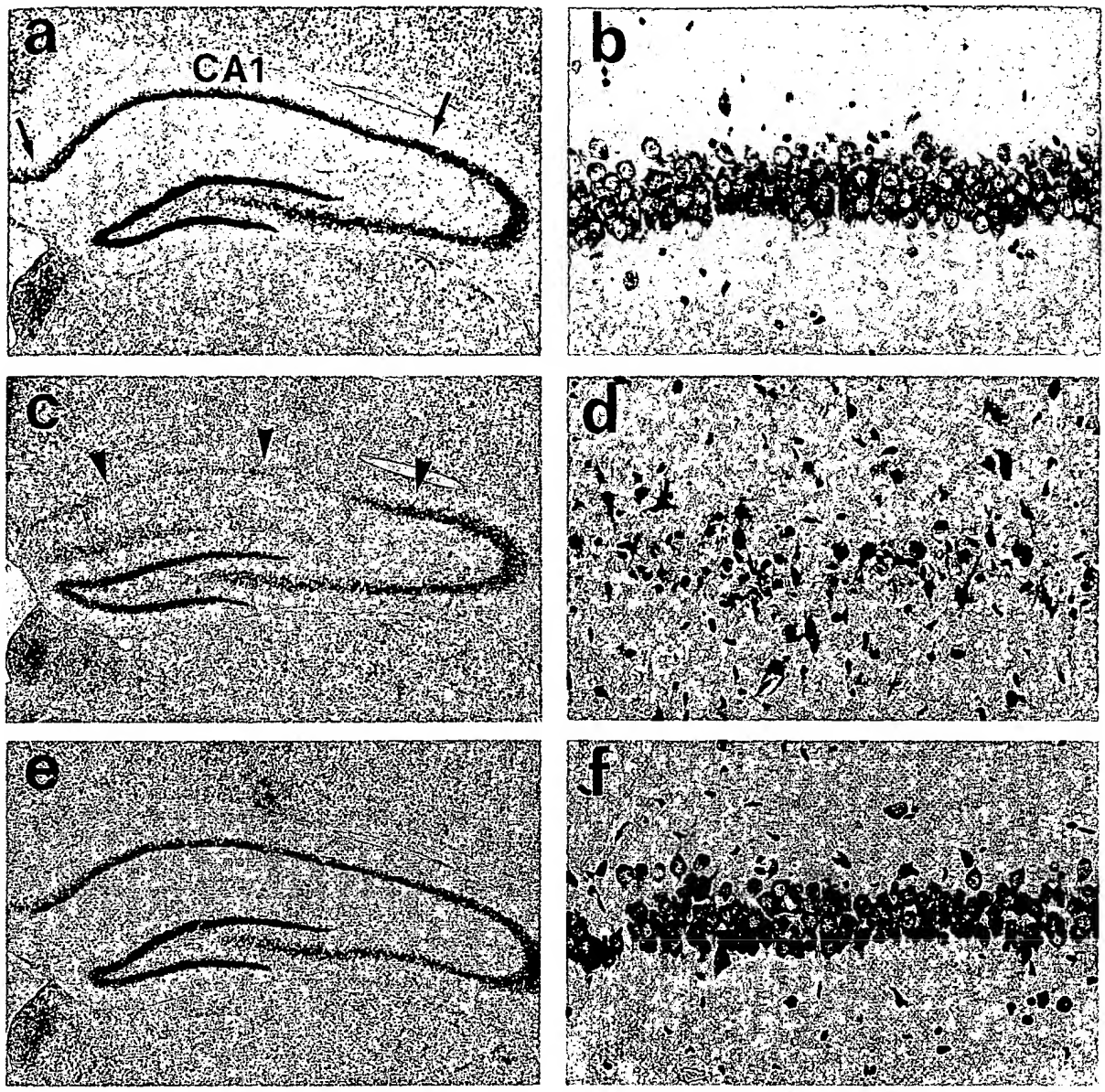

FIG. 1. Representative photomicrographs of hippocampal CA_1 subfield 7 days after 5-min bilateral carotid artery occlusion in gerbils. **a,b:** Sham operation: stratum pyramidale in the CA_1 region (between the arrows). CA_1 pyramidal cells are well preserved under high magnification (b). **c,d:** Vehicle-treated ischemia. Note marked damage to the CA_1 pyramidal cells (arrowheads). **e,f:** Staurosporine (10 ng) treatment. Most of the CA_1 neurons are preserved. Cresyl violet; ×7.5 (a,c,e), ×75 (b,d,f).

markedly phospholipid/Ca^{2+}-dependent PKC in the rat brain (Tamaoki et al., 1986). The IC_{50} values of staurosporine against PKC, cyclic AMP–dependent protein kinase, and cyclic GMP-dependent protein kinase were 1.8, 23, and 31 n*M*, respectively. The IC_{50} values of K-252a, another PKC inhibitor, against PKC, cyclic AMP–dependent protein kinase, and cyclic GMP–dependent protein kinase were 33, 72, and 87 n*M*, respectively (Nakanishi et al., 1988). Staurosporine is a more potent inhibitor of PKC than other PKC inhibitors, such as K-252b (Kase et al., 1987), H-7 (Hidaka and Hagiwara, 1987), trifluoperazine (Schatzman et al., 1981), chlorpromazine (Mori et al., 1980), and polymyxin B (Mazzei et al., 1982). K-252a (10 ng) but not K-252b (up to a dose of 100 ng) protected against pyramidal cell loss (Yoshidomi et al., 1989). Taken together, these findings indicate that staurosporine plays its neuroprotective role by the inhibition of PKC. In contrast, cyclic AMP– and cyclic GMP–dependent protein kinase inhibitors KT5720 (K_i = 60 n*M*; Kase et al., 1987) and KT5822 (K_i = 2.4 n*M*; Kase et al., 1987) and the calmodulin inhibitor W-7 (K_i = 12 μM; Hidaka et al., 1979) did not protect against ischemic neuronal damage in the gerbil CA_1 subfield after transient ischemia. These results sug-

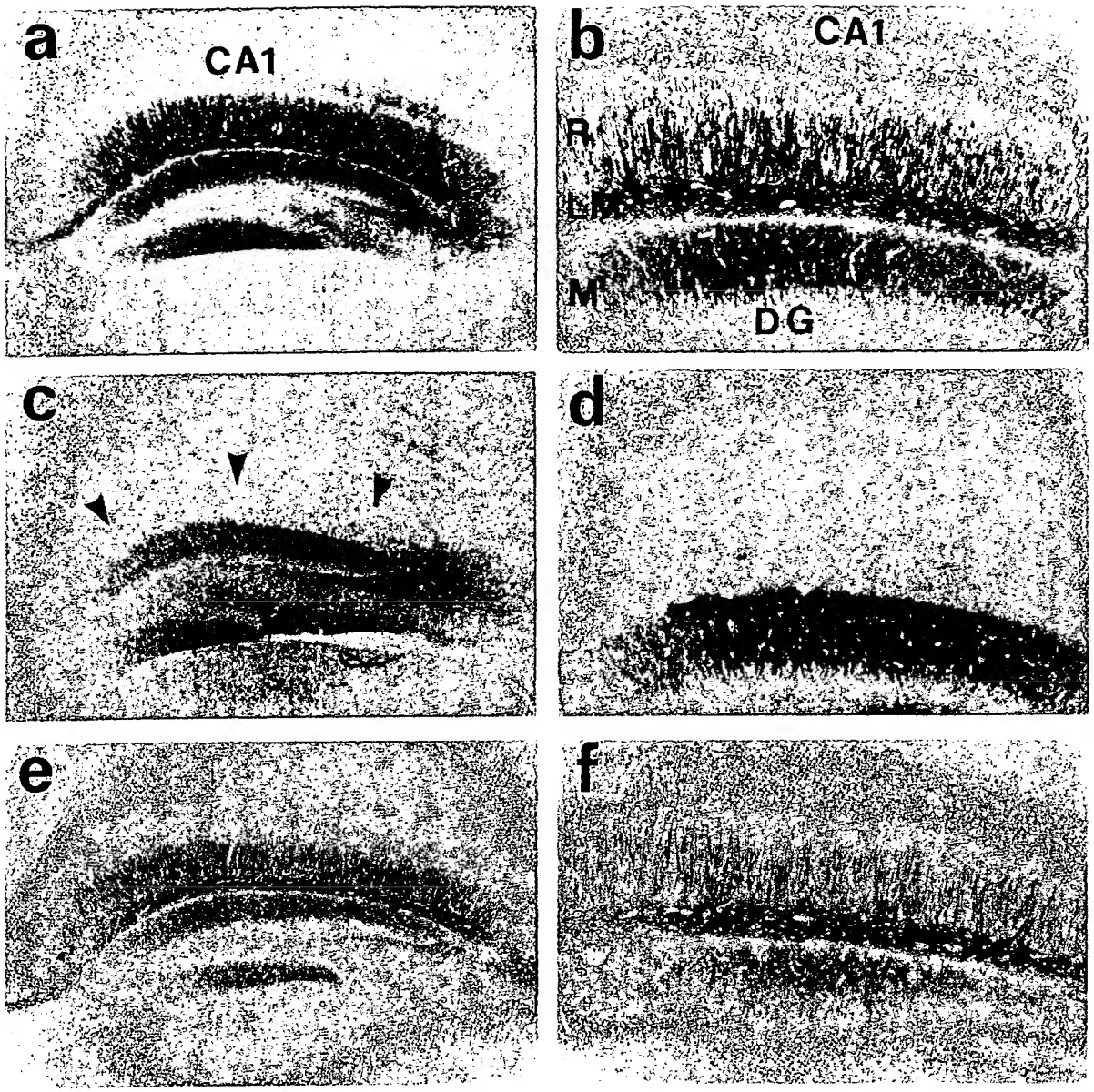

FIG. 2. Representative photomicrographs of the hippocampal CA_1 subfield 7 days after 5-min bilateral carotid artery occlusion in gerbils. **a,b:** Sham operation. **c,d:** Vehicle-treated ischemia. Most of the microtubule-associated protein 2 (MAP2) staining disappeared (arrowheads). **e,f:** Staurosporine (10 ng) treatment. MAP2 immunoreactivity is preserved in the dendritic fields of the CA_1 subfield, indicating the preservation of dendrite arborization of the CA_1 pyramidal cells. DG, dentate gyrus; M, molecular layer of dentate gyrus; LM, stratum lacunosum-moleculare; R, stratum radiatum. MAP2 immunostaining; ×7.5 (a,c,e), ×75 (b,d,f).

gest the possibility that cyclic AMP– and cyclic GMP–dependent protein kinase– and calmodulin-mediated processes did not relate to the mechanisms of the postischemic neuronal damage. Adenylate cyclase molecules, visualized by [^{3}H]-forskolin autoradiography, distributed with low density in the CA_1 subfield (Onodera and Kogure, 1989). However, the possibility exists that KT5720, KT5822, and W-7 could not reach a "therapeutic" intracellular concentration in the present condition.

Marked changes in the intracellular localization of PKC were reported after global ischemia in the near-term rat, showing translocation of PKC from the cytosol to the particulate membrane fraction (Louis et al., 1988). Onodera et al. (1989), using the same rat model as the present study, reported that phorbol ester binding sites in the rat CA_1 subfield increased 1–12 h after the recirculation period. The excitatory amino acid released during and/or after ischemia may play a role in the translocation of PKC, since glutamate can translocate PKC from the cytosol to the membrane fraction (Vaccarino et al., 1987). Moreover, factors affecting enzyme activity and translocation (Kraft and Anderson, 1983; Go-

TABLE 2. *Effect of staurosporine on delayed neuronal death of hippocampal CA_1 neurons in rats*

Treatment	n	Neuronal density (n/mm)	
		Ipsilateral	Contralateral
Sham operation	6	165 ± 18[a]	174 ± 13[a]
Vehicle	9	28 ± 37	22 ± 21
Staurosporine 10 ng			
Pre 30 min	9	78 ± 60[b,c]	36 ± 42
Post 10 min	10	38 ± 54	30 ± 42
Post 3 h	10	34 ± 32	17 ± 15

Staurosporine was injected unilaterally into the CA_1 subfield of the hippocampus 30 min prior to, 10 min after, or 3 h after 20-min forebrain ischemia. Values are means ± SD.

[a] $p < 0.01$ vs. vehicle (Kruskal-Wallis test and two-tailed Mann-Whitney U test).

[b] $p < 0.05$ vs. vehicle (Kruskal-Wallis test and two-tailed Mann-Whitney U test).

[c] $p < 0.001$ vs. contralateral side (paired t test).

palakrishna et al., 1986) are noticed during and after ischemia. Calcium deposits were accumulated in the CA_1 pyramidal cells after ischemia (Simon et al., 1984). Hydrolysis of phosphoinositides into diacylglycerol and inositol phosphate after ischemia (Abe et al., 1987) may also enhance PKC activity. The neuroprotective effect of staurosporine suggests that PKC plays a crucial role in the postischemic modulation of neuronal activity in the hippocampus (Miller, 1986) and in neuronal death of CA_1 pyramidal cells. Staurosporine also reduced dendritic damage of CA_1 pyramidal cells after ischemia. The vehicle-treated ischemia group showed a marked decrease in MAP2 immunoreactivity in the dendritic fields of CA_1 pyramidal cells. In contrast, the MAP2 immunoreactivity of the staurosporine-treated group was similar to that of the sham-operated group. However, the delayed neuronal death of CA_1 pyramidal cells cannot be explained solely by the activation of PKC, because staurosporine cannot protect all CA_1 pyramidal cells. In the rat model, half the pyramidal cells were damaged even though staurosporine was administered before the ischemic insult. Furthermore, we cannot rule out the possibility that other actions of staurosporine, such as inhibition of tyrosine protein kinase (Nakano et al., 1987), played a role in the amelioration of hippocampal CA_1 damage. These indicate that mechanisms other than PKC activation are involved in the generation of CA_1 pyramidal cell necrosis.

Postischemic administration of staurosporine had

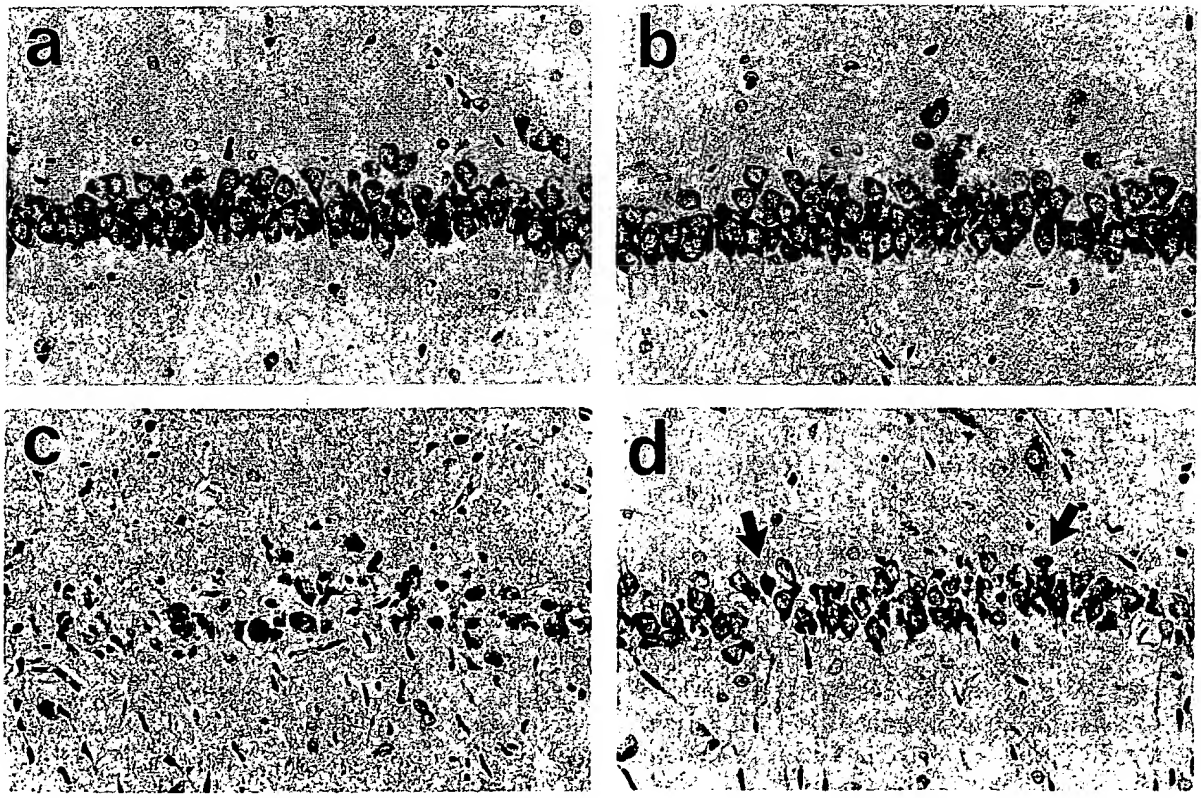

FIG. 3. Representative photomicrographs of the hippocampal CA_1 subfield 7 days after 20-min four-vessel occlusion in rats. **a:** Contralateral to the needle insertion (sham operation). **b:** Ipsilateral to the needle insertion (sham operation). No visible difference is caused by the needle insertion. **c:** Ipsilateral to the vehicle-treated ischemia. The CA_1 pyramidal cells are depleted. **d:** Ipsilateral to the staurosporine (10 ng) treatment 30 min prior to ischemia. The CA_1 neurons are preserved, though some are damaged (arrows). Cresyl violet; ×75.

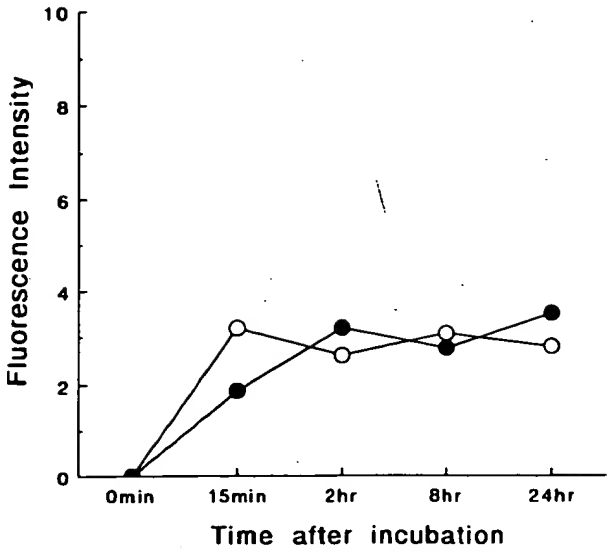

FIG. 4. Time course of cellular distribution of staurosporine after incubation of PC12 cells. ○, intracellular level; ●, extracellular level.

little protective effect for CA_1 pyramidal cells, even if administered immediately after recirculation, although treatment before ischemia was beneficial. This suggests that PKC-mediated processes that occur during and/or shortly after recirculation lead to subsequent events that ultimately determine the fate of pyramidal cells.

In the gerbil, preischemic administration of 10 ng staurosporine protected 82% of the CA_1 pyramidal cells. In contrast, in the rat ischemia model, staurosporine at 10 ng afforded only a 47% neuroprotection of CA_1 pyramidal cells. The distinction in the staurosporine effect may be explained partly by the difference in animal species (Onodera and Kogure, 1988). Since involvement of the hippocampal formation in the seizure activities characteristic of the gerbil has been reported (Paul et al., 1981; Peterson et al., 1985), staurosporine might be more protective in the gerbil by an "anticonvulsive" effect. In general, neuroprotection of postischemic CA_1 neurons by drugs has been more pronounced in gerbil ischemia models than rat models.

In conclusion, preischemic administration of a PKC inhibitor, staurosporine, had a beneficial effect in the protection of the CA_1 pyramidal cells after ischemic insult, both in the gerbil and in the rat, suggesting a contribution of PKC in delayed neuronal death of CA_1 pyramidal cells after ischemia. Cyclic AMP– and cyclic GMP–dependent protein kinase inhibitor and calmodulin kinase inhibitor did not protect CA_1 pyramidal cells in the gerbil. The failure of postischemic staurosporine administration suggests that triggering events leading to the necrosis of CA_1 pyramidal cells occur during the early recirculation period. The mechanisms that finally determine the fate of pyramidal cells remain to be clarified.

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A DERIVATIVE OF STAUROSPORINE (CGP 41 251) SHOWS SELECTIVITY FOR PROTEIN KINASE C INHIBITION AND *IN VITRO* ANTI-PROLIFERATIVE AS WELL AS *IN VIVO* ANTI-TUMOR ACTIVITY

Thomas MEYER[1,3], Urs REGENASS[1], Doriano FABBRO[2], Enrica ALTERI[1], Johannes RÖSEL[1], Marcel MÜLLER[1], Giorgio CARAVATTI[1] and Alex MATTER[1]

[1]*Research Department, Pharmaceuticals Division, CIBA-GEIGY Ltd., CH-4002 Basel; and* [2]*Laboratory of Biochemistry-Endocrinology, Department of Research and Department of Gynecology and Obstetrics, University Clinic Medical School, CH-4031 Basel, Switzerland.*

Analogues of staurosporine were synthesized and their ability to inhibit protein kinases was examined. Staurosporine is a potent but non-selective inhibitor of *in vitro* protein kinase C (PKC) activity (IC_{50} 6.0 nM). The derivative CGP 41 251 had reduced PKC activity with an IC_{50} of 50 nM but showed a high degree of selectivity when assayed for inhibition of cyclic AMP-dependent protein kinase (IC_{50} 2.4 μM), S6 kinase (IC_{50} 5.0 μM) and tyrosine-kinase-specific activity of epidermal growth factor receptor (IC_{50} 3.0 μM). Staurosporine and CGP 41 251 exerted growth inhibition in the human bladder carcinoma line T-24, human promyelocytic leukemia line HL-60 and bovine corneal endothelial cells at concentrations which correlated well with *in vitro* PKC inhibition. In addition, both compounds inhibited the release of H_2O_2 from human monocytes pre-treated with 12-O-tetradecanoyl-phorbol-13-acetate at non-toxic concentrations. *In vivo* anti-tumor activity was examined in T-24 human bladder carcinoma xenografts in athymic nude mice. Tumor growth inhibition tests revealed significant anti-tumor activity ($2p < 0.001$) at 1/10 of the maximum tolerated doses for both compounds. By contrast, a closely related derivative of staurosporine (CGP 42 700) was inactive at concentrations of over 100 μM in all *in vitro* enzyme and anti-proliferative assays as well as in animal tumor models. Our data suggest an association between PKC inhibition and anti-proliferative and anti-tumor activity.

PKC belongs to a group of serine/threonine-specific protein kinases and seems to be ubiquitous in most tissues and organs (Nishizuka, 1986). The enzyme plays an important role in cell-surface signal transduction and controls a wide number of physiological processes including cellular growth and differentiation as well as tumor promotion (Nishizuka, 1984; Anderson *et al.*, 1985). PKC, a phospholipid- and Ca^{2+}-dependent enzyme is activated by diacylglycerol which is formed in response to extracellular signals by turnover of phosphoinositides (Berridge, 1987). Several recent findings indicate an important role for PKC in the regulation of cellular growth. Certain growth factors such as platelet-derived growth factor (PDGF) or interleukin-2 (IL-2) mediate their mitogenic effect in part through the cascade of phosphatidyl inositol hydrolysis (Berridge and Ervine, 1984; Farrar and Anderson, 1985). The generation of diacylglycerol followed by phosphorylation/dephosphorylation-reactions constitutes a series of steps which enable a mitogenic signal, among other pathways, to convey from the plasma membrane to the nucleus. A large number of substrates accepted by PKC have been described (Nishizuka, 1986) but none of them was found to be directly and exclusively growth-related. Nevertheless, recent findings demonstrated the existence of a cellular protein (AP-1) whose transcription stimulatory activity was thought to be mediated *via* phosphorylation by PKC (Lee *et al.*, 1987). In addition, PKC was found to phosphorylate DNA methyltransferase, an enzyme which acts in the nucleus and alters gene expression by changing DNA methylation patterns (DePaoli-Roach *et al.*, 1986). In addition, in *ras*- and *sis*-transformed normal rat kidney cells, elevated levels of diacylglycerol were shown to correlate with persistent activation of PKC (Preiss *et al.*, 1986). One mechanism responsible for the transformed phenotype is thought to be due to alterations in the steady-state levels of diacylglycerol (Fleischman *et al.*, 1986). Furthermore, in fibroblasts transfected with the PKC-1 isozyme, altered gene expression leads to enhanced tumorigenicity (Persons *et al.*, 1988). It is thus hypothesized that increased activity of PKC-1 results in a tumor-promoting effect similar to that of phorbol esters (Diamond *et al.*, 1978) which bind to and activate PKC directly (Castagna *et al.*, 1982).

Several inhibitors of PKC have been detected and are capable of inhibiting PKC-mediated cellular responses (Hidaka and Hagiwara, 1987) such as the expression of ornithine decarboxylase (Jetten *et al.*, 1985) or superoxide generation by cells of the immune system (Bass *et al.*, 1987). Among them, staurosporine, an alkaloid isolated from microbial sources (Omura *et al.*, 1977), exerted the most potent inhibitory effect on PKC activity but revealed non-selective inhibition. The compound exerted anti-proliferative effects against various cell lines *in vitro* (Tamaoki *et al.*, 1986).

In an attempt to elucidate in more detail the role of PKC in cellular growth regulation, structural analogues of staurosporine were synthesized, aiming at higher selectivity. The *in vitro* and *in vivo* effects of one such derivative were compared with its biologically inactive analogue as well as its parent compound staurosporine.

MATERIAL AND METHODS

Compounds (staurosporine, CGP 41 251, CGP 42 700) were fermented and synthesized respectively by CIBA-GEIGY research laboratories. Their chemical structures are shown in Figure 1. The compounds were dissolved in DMSO (10 mM) and stored at −20°C. Dilutions were freshly made in DMSO/water 1:1 and the final concentration of DMSO in the enzyme assays was <0.5%. All reagents used with no specific indication were obtained from Sigma, St. Louis, MO.

Purification of PKC

PKC was purified from porcine brain using DEAE-cellulose

[3]To whom reprint requests should be sent.

Abbreviations: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; EDTA, ethylenediaminetetraacetic acid diosodium salt; EGTA, ethyleneglycol bis (β-aminoethyl ether)-N'N'N'N'-tetraacetic acid; MES, 2[N-morpholino] ethanesulfonic acid; HEDTA, N-(2-hydroxethyl) ethylenediamine, N'N'N'N' triacetic acid trisodium; MOPS, 4-morpholinepropanesulfonic acid; TRIS, Tris (hydroxymethyl) aminomethane; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; DTT, α-dithiothreitol; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate; MTD, maximum tolerated dose; PKC, protein kinase C; CEC, bovine corneal endothelial cells.

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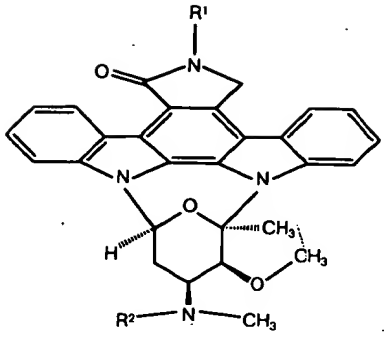

FIGURE 1 – Structures of staurosporine and its derivatives. R1 = R2 = H: Staurosporine; R1 = H, R2 = Benzoyl: CGP 41 251; R1 = Benzyl, R2 = Benzoyl: CGP 42 700.

chromatography followed by affinity chromatography on polyacrylamide-immobilized phosphatidylserine exactly as described by Uchida and Filburn (1984) and modified by Borner *et al.* (1987). The enzyme activity was stabilized with 1 mg/ml of trypsin inhibitor (Boehringer, Mannheim, FRG) and dialyzed against 0.1 mM EDTA and 20 mM TRIS-HCl, pH 7.4, before use. PKC displayed a specific activity of 5,000 U/mg. One unit of PKC activity was defined as the amount of enzyme transferring one nmole ^{32}P from γ-[^{32}P]-ATP (Amersham, Little Chalfont, UK; 10 Ci/mol) to histone H1 (Sigma, type V-S) in 1 min/mg protein.

Assay of PKC

PKC activity was assayed essentially as described by Fabbro *et al.* (1985) and carried out in a reaction mixture containing in a final volume of 100 µl, 20 mM TRIS-HCl, pH 7.4, 200 µg/ml histone H1 (type V-S), 300 µM $CaCl_2$, 10 mM $Mg(NO_3)_2$, 10 µg/ml phosphatidylserine, 1 µg/ml diolein, 24 µM ATP (0.25 µCi γ-[^{32}P]-ATP), 2.5 U of purified enzyme and various concentrations of inhibitors. Aliquots of 50 µl were analyzed for substrate phosphorylation (Witt and Roskoski, 1975) using P81 chromatography paper (Whatman, Maidstone, UK). Measurements of enzyme inhibition in all kinase assays were performed in at least 2 independent experiments. Each single-point determination represents the mean of triplicates with standard deviation ≤10%.

Assay of cAMP-dependent protein kinase

Isolation and purification of the catalytic subunit of cAMP-dependent protein kinase was performed by Dr. B. Hemmings, F. Miescher Institute, Basel, Switzerland, and kindly provided as a dilution of 400 U/ml. The activity of cAMP-dependent protein kinase was measured (Reimann and Beham, 1983) in a reaction mixture of 100 µl. It contained, in final concentrations, 70 mM MES-NaOH, pH 6.9, 75 mM NaCl, 0.5 mM EDTA, 2.5 mM Mg-acetate, 1.2 mg/ml bovine serum albumin, 0.1 mg/ml Kemptide (Bachem, Bubendorf, Switzerland), 0.01 U of purified enzyme and various concentrations of compounds. The mixture was pre-incubated for 2 min at 32°C and the reaction initiated by adding pre-warmed 1 mM ATP (0.5 µCi γ-[^{32}P]-ATP. Aliquots of 50 µl were analyzed for substrate phosphorylation using P81 chromatography paper as collecting material.

Assay of phosphorylase kinase

Effects of compounds on the activity of phosphorylase kinase were determined using the method of Cohen (1983). A 100-µl volume of reaction mixture contained 60 mM Na-glycerophosphate pH 7.0, 0.05% 2-mercaptoethanol, 25 mM TRIS-HCl pH 6.8, 0.6 mM HEDTA, 0.9 mM $CaCl_2$, 3.3 mM Mg-acetate, 5 U of phosphorylase kinase from rabbit muscle (500 U/mg protein) 1.25 mg/ml phosphorylase b and compounds at different concentrations. After pre-incubation for 2 min at 32°C, pre-warmed 1 mM ATP (0.5 µCi γ-[^{32}P]-ATP) was added to start the reaction. Termination was achieved by spotting 50 µl aliquots on 31 ET chromatography paper (Whatman) and subsequent transfer into ice-cold 10% trichloroacetic acid.

Assay of the tyrosine-specific phosphorylation of epidermal growth factor (EGF) receptor

The EGF receptor was isolated from membranes of A431 cells (House *et al.*, 1984) and enzymatic activity determined (House *et al.*, 1984) with minor modifications using angiotensin II as substrate. The reaction mixture (50 µl) contained 5 µl of A431 membrane preparation (approx. 50 µg protein), 10 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM Na_3-orthovanadate, 0.1% Triton X-100, 13.5 µM γ-[^{32}P]-ATP, 1 µM epidermal growth factor, 50 µg (1 mg/ml) angiotensin II and various concentrations of compounds. The membranes were pre-incubated in the presence or absence of EGF for 10 min on ice. The reaction was started by the addition of γ-[^{32}P]-ATP, and incubation continued for 5 min at 37°C. The reaction was stopped by adding 100 µl of 10% trichloroacetic acid and left for 30 min on ice. After 5 min centrifugation at 1,500 *g*, aliquots of the supernatant were spotted on P81 chromatography paper and the filter paper was washed 5 times in 6% acetic acid. The filter papers were dried and radioactivity was measured in a liquid scintillation counter.

Assay of S6 kinase

Highly purified S6 kinase (4,000 U/ml) and 40 S ribosomal subunit were a gift from Dr. G. Thomas, F. Miescher Institute. The enzyme was purified and enzyme activity was measured as described by Jenö *et al.* (1988). The activity of S6 kinase was measured using the 40S ribosomal subunit from rat liver. In a final volume of 50 µl the reaction mixture contained 50 mM MOPS, pH 7.0, 1 mM DTT, 10 mM $MgCl_2$, 30 µM γ-[^{32}P]-ATP (10–30 cpm/fmol), 10 mM p-nitrophenylsulfate, 17 µg 40S subunit, various amounts of test compounds and 5 µl of enzyme fraction diluted 1:10 with Mono S buffer. The reaction was performed at 30°C and terminated after 30 min by the addition of 50 µl of SDS-stop solution. Phosphorylated S6 was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. S6 kinase activity was quantified by cutting out the S6 band from the dried gels followed by liquid scintillation counting.

Isolation of monocytes and H_2O_2 release test

Human blood monocytes were isolated from leukapheresis blood samples by counter-current centrifugation elutriation (Stevenson *et al.*, 1983) with slight modifications. The cells were on average 96% pure as assessed by morphology. After elutriation, monocytes were resuspended in RPMI 1640 medium containing human AB serum (Blood Bank, Berne, Switzerland), plated in 96-well plates at 10^6 per ml, 100 µl/well, incubated for 2 hr and washed. H_2O_2 formation, its monitoring and quantification were performed exactly as described by De la Harpe and Nathan (1985). Briefly, the reaction mixture was added containing 100 nM TPA in the presence or absence of test compounds. Release of H_2O_2 was measured in triplicate, each single point recording at 0, 15, 30 and 60 min the horseradish-peroxidase-catalyzed oxidation of fluorescent scopoletin by H_2O_2 in a microfluorimeter. The total amount of H_2O_2 released/well/hr was calculated using a correction formula for the fluorescence changes in the cell-free wells.

Antiproliferative assays

T-24 human bladder carcinoma cells were obtained from Dr.

L.J. Old, Sloan Kettering Institute, New York, and cultured in Eagle's minimal essential medium (Gibco, Paisley, UK) supplemented with 5% fetal bovine serum. HL-60 promyelocytic leukemia cells purchased from the American Type Culture Collection, Rockville, MD, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco). Bovine corneal endothelial cells were established from calf eyes (Gospodarowicz *et al.*, 1977) and grown in the presence of fibroblast growth factor (Seragen, Boston, MA or Biomedical Technologies, Staughton, MA). Cells (T-24: 10^3, HL-60: 7.5×10^3, CEC: 5×10^3) were seeded into 96-well microtiter plates and incubated overnight. Drugs were added in serial dilutions on day 1. The plates were incubated for 5 to 6 days in the case of T-24 and CEC cells, or 8 days in the case of HL-60 cells. This allowed the control cultures to undergo at least 3 cell divisions. After incubation, T-24 and CEC cells were fixed with 3.3% glutaraldehyde, washed with water and stained with 0.05% methylene blue. After washing, the dye was eluted with 3% HCl and the optical density per well measured with a Titertek multiskan at 665 nm. HL-60 cell growth was monitored by using the calorimetric MTT (tetrazolium) assay (Mosmann, 1983). After the incubation period, cells were washed with phosphate-buffered saline in the microtiter plate. DMSO was then added to each well and the dishes were put on a shaker for 20 min. Optical density was measured at 540 nm. IC_{50} values were evaluated by a computerized system using the formula: (OD Test − OD Start)/(OD Control − OD Start) ×100. IC_{50} was defined as the drug concentration which leads to 50% of cells per well compared to control cultures (100%) at the end of the incubation period. IC_{50} values represent the mean of at least 2 independent assays using serial dilutions of the drug originally dissolved in DMSO and diluted with culture medium.

In vivo *anti-tumor activity*

For MTD determinations, 3 animals per dose group were treated once i.p. The dosage was increased until animal death occurred within 10 days after drug treatment. *In vivo* anti-tumor activity was tested using T-24 bladder carcinoma xenografts in athymic nude mice. To measure growth inhibition, test animals were treated i.p. with 1/20 to 1/10 of the MTD starting at days 3–4 after implantation of the tumor (day 0) until day 24–25 (5 times weekly). In regression tests, the treatment (1/20–1/10 of MTD daily) was started at days 10–11 after transplantation when the tumor reached a diameter of 7–9 mm. Treatment was continued for 10 days. Tumor growth was followed by measuring 2 perpendicular tumor diameters. Tumor volumes were calculated according to the formula ($\pi \times L \times D^2/6$), where L represents the largest diameter and D the tumor diameter perpendicular to L (Evans *et al.*, 1982). Stable disease was defined as a stop in tumor growth during the course of treatment.

Data were statistically analyzed using the test method of Dunnett (1955).

Other analytical methods

Protein content was determined by the standard method (BioRad, Munich, FRG) (Bradford, 1976) using bovine serum albumin as standard.

RESULTS

Effect on protein kinase activities in vitro

Staurosporine as well as CGP 41 251 caused a concentration-dependent inhibition of PKC. IC_{50} values determined by linear regression analysis were 6.0 nM and 50.0 nM, respectively (Table I). In contrast to staurosporine, CGP 41 251 revealed a high degree of selectivity when assayed for additional protein kinases. As summarized in Table I, IC_{50} values obtained with CGP 41 251 were 50 times higher in the case of cAMP-dependent protein kinase, 100 times higher for S6 kinase and 60 times higher for the tyrosine kinase activity of the EGF receptor. No selectivity was seen when CGP 41 251 was tested for inhibition of phosphorylase kinase. CGP 42 700, which shares structural similarities with CGP 41 251 and staurosporine, was inactive ($IC_{50} > 100$ μM) in all enzyme assays.

TABLE I – EFFECTS ON PROTEIN KINASE ACTIVITIES *IN VITRO*

Compounds	*In vitro* enzyme inhibition (IC_{50}, μM)				
	PKC	PKA	PK	S6K	TPK
Staurosporine	0.006	0.015	0.003	0.005	0.025
CGP 41 251	0.050	2.4	0.048	5.0	3.0
CGP 42 700	>100	>100	>100	>100	>100

PKC = protein kinase C; PKA = cyclic AMP-dependent protein kinase; PK = phosphorylase kinase; S6K = S6 kinase; TPK = tyrosine-specific protein kinase of epidermal growth factor receptor.

In vitro *anti-proliferative effect*

Table II summarizes the results demonstrating that both CGP 41 251 and staurosporine were anti-proliferatively active. CGP 41 251 was 3–9 times less potent than staurosporine depending on the cell lines used, whereas CGP 42 700 was inactive at concentrations >1,000 times higher than staurosporine. Comparison of the anti-proliferative effects revealed no major differences in sensitivity among different cell lines.

Effect on H_2O_2 release from human monocytes

CGP 41 251 and staurosporine were assayed for inhibition of active phorbolester-induced formation of hydrogen peroxide by human monocytes. The release of H_2O_2 by TPA-treated monocytes was time-dependent and reached maximum levels after 60 min (approx. 150 nmoles H_2O_2/mg protein). In the presence of staurosporine or CGP 41 251, the release was decreased in a concentration-dependent manner (Table III). Half maximum inhibition was observed at around 40 nM and 150 nM for staurosporine and CGP 41 251, respectively. In contrast, the inactive analogue (CGP 42 700) tested in parallel did not affect the release of H_2O_2 from these cells.

In vivo *anti-tumor effects*

Initially, the MTD of a single administration was determined. In contrast to staurosporine, the MTD of CGP 41 251 was approx. 250 times higher. When treatment was started 3 days after implantation of T-24 tumor cells of athymic nude mice, staurosporine exerted significant ($2p < 0.001$) anti-tumor effects (Table IV) at 1/10 of MTD but not at 1/20 of MTD. In contrast, CGP 41 251 showed significant growth inhibition ($2p < 0.001$) at 1/10 and 1/20 of MTD. Figure 2 shows that anti-tumor activity with 1/10 of MTD occurred even when treatment was delayed until 10 days after tumor transplantation. CGP 41 251 was more potent than staurosporine and weak anti-tumor effects of CGP 41 251 could still be detected at 1/20 of MTD. The *in vitro* inactive compound CGP 42 700 was also inactive in all *in vivo* experiments.

TABLE II – PKC INHIBITORS: ANTI-PROLIFERATIVE EFFECTS *IN VITRO* (IC_{50} VALUES, μM)

Cell line	Staurosporine	CGP 41 251	CGP 42 700
T-24	0.029	0.211	>76
HL-60	0.13	0.30	63.6
CEC	0.022	0.197	>76

T-24 = human bladder carcinoma line; HL-60 = human promyelocytic leukemia line; CEC = bovine corneal endothelial cells.

DISCUSSION

The formation of diacylglycerol and the subsequent activation of PKC have been demonstrated for a variety of growth-stimulatory processes (Nishizuka, 1986). We have therefore attempted to test the hypothesis that inhibition of PKC activity could lead to growth inhibiton. Potent kinase inhibitors with high selectivity for PKC inhibition had to be identified and evaluated. Among the high number of described protein kinases (Hunter, 1987) involved in signal transduction mechanisms or in growth-regulatory processes, a few with specificity for the phosphorylation of either serine/threonine or tyrosine residues were included in compound testing. A staurosporine derivative (CGP 41 251) with a high degree of selective inhibition for PKC, anti-proliferative and anti-tumor activity has been identified.

The comparison between staurosporine and CGP 41 251 showed that the chemical modification of staurosporine at the secondary amine (Fig. 1) caused slightly reduced potency in PKC inhibition (approx. 8 times) but led to a new chemical structure exerting a high degree of selectivity (IC_{50} values 50–100 times higher than PKC). Among 4 kinases tested, phosphorylase kinase, an enzyme participating in glycogen metabolism, was inhibited by CGP 41 251 at a concentration range comparable to that of PKC. From its known biological function, phosphorylase kinase inhibition is not considered to play a role in growth regulation. On the other hand, this finding implicates the possible inhibition of other kinases not yet included in our specificity assays. Attempts to increase the specificity of staurosporines should be considered.

Recently, molecular cloning and sequence analysis of PKC were achieved, revealing the existence of a gene family coding for several closely related but distinct polypeptides (Parker *et al.*, 1986; Ono *et al.*, 1988). Preliminary data suggest a different distribution of each subspecies among various tissues and cells (Ono *et al.*, 1987; Kosaka *et al.*, 1988). In this report, PKC inhibition studies were all performed with highly purified PKC from porcine brain but this preparation was not analyzed for its content in the various PKC subspecies. It remains to be shown which subspecies are involved in growth control and affected by staurosporine and its more specific derivative CGP 41 251.

To test whether inhibition of PKC at the enzyme level is also reflected by inhibition of a PKC-mediated cellular function, the release of H_2O_2 from human monocytes was measured. The response to TPA, which activates PKC directly, could be monitored within a few minutes. Our data demonstrate that the inhibition of this rapid PKC-specific signal transduction is highly correlated with the PKC-inhibiting potencies of the drugs. This indicates that the drugs are able to modify PKC-mediated functions intracellularly.

When assayed for anti-proliferative activity, staurosporine was 3–9 times more potent than CGP 41 251. Data revealed good correlation between potency of PKC inhibition and anti-proliferative activity of the compounds. Even though 3 totally different target cells were selected, the sensitivity of the cells to the kinase inhibitors was uniform. The increased specificity of CGP 41 251 has so far not revealed a cell-type-specific anti-proliferative effect. IC_{50} values for growth inhibition of

TABLE III – EFFECT ON H_2O_2 RELEASE FROM HUMAN MONOCYTES

Concentration	H_2O_2 release (nmoles H_2O_2/mg protein)		
	Staurosporine	CGP 41 251	CGP 42 700
0	154	154	154
1 nM	190	154	149
10 nM	198	177	157
100 nM	10	68	171
1 μM	10	3	173
10 μM	0	3	181

Standard error ≤ 10%.

TABLE IV – ANTI-TUMOR EFFECTS OF STAUROSPORINE AND DERIVATIVES AGAINST T-24 HUMAN BLADDER CARCINOMA TRANSPLANTED INTO FEMALE CD-1 NUDE MICE

Compound	MTD[2] mg/kg	Dose mg/kg	Dose MTD[2]	Growth inhibition T/C%
Control				100
Staurosporine	1	0.1	1/10	64[1]
		0.05	1/20	94
CGP 41 251	250	25.0	1/10	33[1]
		12.5	1/20	60[1]
CGP 42 700	500	100	1/5	88
		50	1/10	93

[1] $2p \leq 0.001$ (Dunnet test).–[2]MTD = maximum tolerated dose.

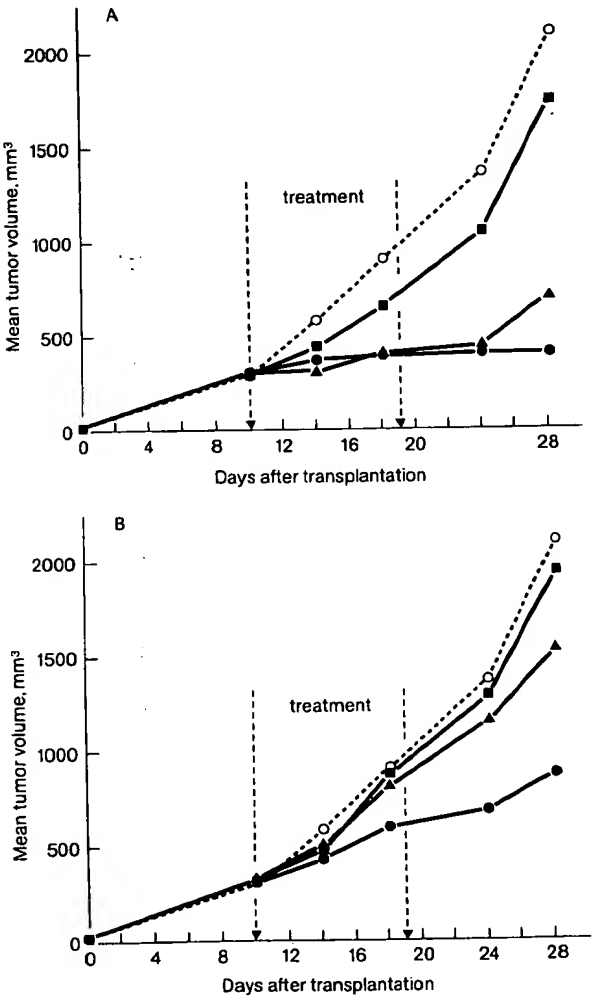

FIGURE 2 – *In vivo* anti-tumor activity against T-24 bladder carcinoma xenografts in athymic nude mice. Animals were treated with 1/10 of MTD (*a*) or with 1/20 of MTD (*b*) between days 10 to 19 with staurosporine (▲), CGP 41 251 (●), CGP 42 700 (■) or vehicle (○). Similar data were obtained in 2 independent experiments.

HL-60 cells by staurosporine exceeded IC_{50} value for PKC inhibition by about 20 times.

In vivo, staurosporine and CGP 41 251 were active in the growth inhibition model and caused stable disease in the regression test at tolerated doses. UCN-01, an agent isolated from *Streptomyces* and chemically related to staurosporine, is equally potent but more selective for PKC inhibition than staurosporine and possesses weak anti-tumor activity against murine lymphatic leukemia P388 (Takahashi *et al.*, 1987). In contrast to CGP 41 251, the chemically related compound CGP 42 700 was inactive in all assays. The increased specificity for PKC inhibition led to increased compound tolerability (MTD) and increased anti-tumor effect at equally tolerated doses. The loss of activities against protein kinases other than PKC did not obviously lead to loss of anti-tumor activities. In contrast, our data indicate a higher therapeutic index for CGP 41 251 in comparison to staurosporine. It still remains to be demonstrated whether the anti-proliferative as well as the anti-tumor activity exerted by CGP 41 251 was exclusively due to deregulation of PKC activity.

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